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Identification of *Lotus* Rhizobia by Direct DNA Hybridization of Crushed Root Nodules

J. E. COOPER,* A. J. BJOURSON, AND J. K. THOMPSON

Agricultural and Food Bacteriology Research Division, Department of Agriculture for Northern Ireland, Belfast BT9 5PX, Northern Ireland

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Hybridization of crushed *Lotus pedunculatus* root nodules with ^{32}P-labeled total genomic DNA probes was used to identify *Rhizobium loti* and *Bradyrhizobium* sp. (*Lotus* rhizobia). Probes always hybridized with homologous target DNA and frequently with DNAs of other strains from the same genus. Intergeneric hybridization did not occur. Results were comparable to those from colony hybridization.

Identification of *Rhizobium* strains in legume root nodules by means of DNA colony hybridization, first reported by Hodgson and Roberts (4), offers potential advantages over immunological and antibiotic resistance-marking techniques by reducing cross-reactions and permitting studies with genetically unmodified strains. Working with *Rhizobium leguminosarum* biovar *trifolii* and using a modification of a technique originally developed for the detection of cloned DNA sequences in plasmids (3), Hodgson and Roberts (4) found that total genomic DNA probes demonstrated a high degree of generic and strain specificities. More recently, the same technique was used to differentiate *R. fredii*, *R. leguminosarum*, and *Galega* rhizobia from other *Rhizobium* species (8).

This report concerns the use of DNA-DNA hybridization for identifying *Lotus* rhizobia in a study which had two objectives: (i) to develop a simplified hybridization procedure based on direct probing of crushed root nodules, thereby eliminating the culturing step required for colony hybridization, and (ii) to ascertain the suitability of total genomic DNA probes for identification of fast- and slow-growing rhizobia from *Lotus pedunculatus*. Results are compared with those from colony hybridization, and the limitations of total genomic DNA probes for strain differentiation in rhizobia are discussed.

Total genomic DNA probes were constructed for three strains of *Rhizobium loti* (NZP2014, NZP2037, and NZP2042) and two strains of *Bradyrhizobium* sp. (*Lotus* rhizobia) (F79 and CC814s) (Table 1) by the extraction and purification procedures described by Hodgson and Roberts (4) with the following modifications: rhizobia were grown in S10 broth (7) to minimize extracellular polysaccharide production, and lysates were digested with RNase A (200 μg ml^{-1}) at 37°C for 30 min prior to phenol extraction. DNA was labeled with [α-^{32}P]dCTP by nick translation (6) to achieve a specific activity of 10^8 cpm μg of DNA^{-1}.

For colony hybridization, sterile nitrocellulose filters (HAHY; Millipore Corp.) were inoculated with pure cultures of each strain and transferred immediately to the surfaces of yeast-mannitol agar plates, which were then incubated at 26°C for 5 days to allow colony development. Colonies were lysed from below by placing the filters onto Whatman 3MM filter paper saturated with a 10% solution of sodium dodecyl sulfate (SDS) containing proteinase K (200 μg ml^{-1}) and incubating the filters at 37°C for 1 h. Liberated DNA was denatured, neutralized, and dried at 80°C for 2 h (5).

For crushed root nodule hybridization, nodules were harvested from *L. pedunculatus* seedlings which had been inoculated with single strains of rhizobia and grown in nitrogen-free rooting solution (pH 6.7) under aseptic conditions (1). A further set of nodules was harvested from plants which had been grown at pH 4.6 and inoculated with a 1:1 mixture of *R. loti* NZP2014 and *Bradyrhizobium* sp. strain F79. After surface sterilization in 5% H_2O_2 and washing in sterile water, nodules were individually crushed in 40 μl of sterile deionized water. Crushed root nodules formed by single-strain inocula were spotted in 40-μl volumes onto a single nitrocellulose filter, and those formed by the mixed-strain inoculum were spotted in 20-μl amounts onto duplicate nitrocellulose filters. Sheets of Whatman 3MM filter paper were placed beneath the filters to draw fluid downwards and concentrate cells on the surfaces of the filters. The procedures for cell lysis and the denaturation, neutralization, and drying of liberated DNA were as described above for colony hybridization.

Filters were rewetted from below with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) for 10 min at 20°C, followed by washing in a solution of 50 mM Tris (pH 8.0), 1 M NaCl, 1 mM EDTA, and 0.1% SDS for 60 min at 57°C. Prehybridization was performed by shaking the filters gently for 4 to 5 h at 57°C in heat-sealed plastic bags containing a solution (0.2 ml cm of filter^{-2}) of 4× SSC, 40% formamide, 0.1% SDS, and denatured salmon sperm DNA (100 μg ml^{-1}). Hybridization was performed in the same volume of fresh prehybridization solution supplemented with denatured (boiled for 5 min) probe DNA (ca. 10^6 cpm ml^{-1}) at 57°C for 12 to 16 h. Filters were washed for 1 h at 65°C with four changes of a solution of 2× SSC and 0.1% SDS, followed by a single wash for 1 h in a solution of 0.1% SSC and 0.5% SDS at the same temperature. After being dryed, filters were subjected to autoradiography at −70°C with Kodak X-OMAT RP4 film and Du Pont Cronex Lightning-Plus intensifying screens. Exposure times for the production of satisfactory signals ranged from 2 to 24 h, the longer times being required for crushed root nodule hybridization.

Results from colony hybridization (Fig. 1a) indicated that *R. loti* probes did not hybridize with *Bradyrhizobium* target

* Corresponding author.

TABLE 1. Details of rhizobia

Organism	Original host plant	Effectiveness on *L. pedunculatus*	Source[a]
R. loti NZP2037	*L. divaricatus*	Effective	DSIR, Palmerston North, New Zealand
R. loti NZP2014	*L. corniculatus*	Effective	DSIR
R. loti NZP2042	*L. pedunculatus*	Effective	DSIR
Bradyrhizobium sp. strain CC814s	*L. hispidus*	Highly effective	Division of Plant Industry, CSIRO, Canberra, Australia
Bradyrhizobium sp. strain F79	*L. pedunculatus*	Highly effective	Northern Ireland pasture soil via plant infection dilution assay

[a] DSIR, Department of Scientific and Industrial Research; CSIRO, Commonwealth Scientific and Industrial Research Organization.

DNA and that *Bradyrhizobium* probes did not hybridize with *R. loti* target DNA. However, within each genus probes were not strain specific, and all probes except *R. loti* NZP2037 hybridized strongly with DNAs of other strains. This result could not be eliminated by increasing stringencies at the hybridization or washing stage. In some cases a probe-target reaction did not correspond to the reciprocal hybridization reaction (e.g., NZP2037 probe-NZP2042 target and vice versa). This feature of colony hybridization has been noted by other workers (4) and was attributed to variations in the binding of DNA to the filter, the size of colonies, or the activity of probes.

In crushed root nodule hybridization (Fig. 1b) probes again hybridized only with DNAs of strains from the same genus. Signals achieved with homologous probe-target DNA combinations were comparable to those found in colony hybridization, but differences in signal intensity indicated variations in the quantity of bacterial DNA in root nodules. A lack of strain specificity, although less pronounced, was again evident within the *R. loti* and *Bradyrhizobium* strain groups, but this pattern differed in comparison with that found in colony hybridization. As with colony hybridization, probe specificity at the strain level could not be improved by increasing stringencies.

The specificity of the probes was used to identify *Bradyrhizobium* sp. (strain F79) or *R. loti* (strain NZP2014) in crushed root nodules from *L. pedunculatus* which had been inoculated with a mixture of these strains in a 1:1 ratio (Fig. 2). Of 25 crushed root nodules, 16 hybridized exclusively with the NZP2014 probe, 5 hybridized solely with the F79 probe, and 2 hybridized with both probes (indicating joint nodule occupancy). Two crushed root nodules produced no signal with either probe, perhaps because there was too little DNA in the sample or because of a loss of DNA from the filter at the hybridization or washing stage.

In this study the intensity of the autoradiographic signal produced in crushed root nodule hybridization was comparable to that produced in colony hybridization, and the culturing step required for colony formation could therefore be omitted for the identification of rhizobia in legume root nodules. Our results suggest that total genomic DNA probes cannot reliably distinguish between closely related rhizobia but are suitable for distinguishing between rhizobia with a low percent DNA homology (*R. loti* and *Bradyrhizobium* sp. show less than 6% homology). In this respect our results concur with those of Wedlock and Jarvis (8), who found that total genomic DNA probes from a number of *Rhizobium* species hybridized strongly with DNAs of closely related strains but not with DNAs of distantly related strains. This technique would, therefore, be suitable for studying competition for nodulation of *Lotus* spp. between known strains of *R. loti* and *Bradyrhizobium* sp. Differentiation within groups of closely related *Rhizobium* strains would require the construction of probes from cloned DNA fragments which are unique to the strain of origin. This approach has previously been used to produce *Salmonella*-specific probes (2).

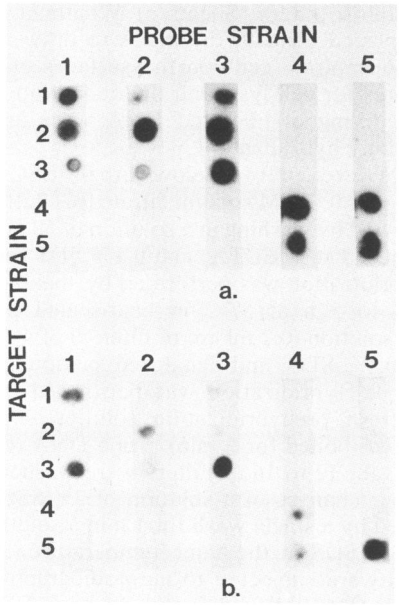

FIG. 1. Hybridization of ^{32}P-labeled total genomic DNA probes with colonies (a) and with crushed root nodules from *L. pedunculatus* inoculated with *R. loti* or with *Bradyrhizobium* sp. (b). Strain codes: 1, NZP2014; 2, NZP2037; 3, NZP2042; 4, CC814s; 5, F79. Strains 1, 2, and 3 were *R. loti*, and strains 4 and 5 were *Bradyrhizobium* sp. Filters with colonies and crushed root nodules were exposed to X-ray film for 6 and 20 h, respectively.

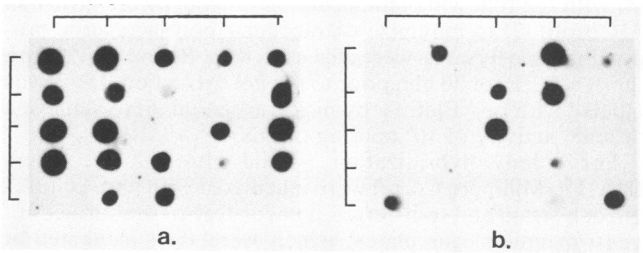

FIG. 2. Hybridization of crushed root nodules from *L. pedunculatus* inoculated with a 1:1 mixture of *R. loti* NZP2014 and *Bradyrhizobium* sp. strain F79. Twenty-five crushed root nodules were spotted onto duplicate filters and hybridized to ^{32}P-labeled total genomic DNA probes from strains NZP2014 (a) and F79 (b). Filters were exposed to X-ray film for 24 h.

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